Journal of Medical Virology 58:426–434 (1999)

Genomic Diversity of "Norwalk Like Viruses" (NLVs): Pediatric Infections in a Brazilian Shantytown

Christine G. Parks,[1] Christine L. Moe,[1]* Deanne Rhodes,[1] Aldo Lima,[3] Leah Barrett,[2] Fanchen Tseng,[1] Ralph Baric,[1] Andrew Talal,[1] and Richard Guerrant[2]

[1]*Department of Epidemiology, School of Public Health, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina*
[2]*Department of Geographic Medicine, University of Virginia, Charlottesville, Virginia*
[3]*Clinical Research Unit, Division of Infectious Diseases, Federal University of Ceara, Fortaleza, Brazil*

"Norwalk-like viruses" (NLVs) are a common cause of epidemic gastroenteritis in adults and children in developed countries. However, little is known about the role of NLVs in endemic pediatric gastroenteritis in developing countries. We sequenced Genogroup I and II NLV reverse transcription-polymerase chain reaction (RT-PCR) products from an 81-nucleotide region of the viral RNA polymerase gene to examine the molecular epidemiology of NLV infection in children younger than 5 years of age in Forteleza, Ceará, Brazil. NLV-positive PCR products were obtained from stool specimens collected over a 16-month period (1990–1991) from diarrhea cases and controls in a cohort of 120 children in an urban shantytown and from a study in the same city of hospitalized children with persistent diarrhea. Eight unique strains were detected in 15 specimens from 10 cohort children and in two hospital specimens. Nucleotide identity between the strains (5 Genogroup I, 3 Genogroup II) ranged from 63% to 88%. We designated these strains BraV1–8, for Brazil virus 1–8. The degree of genomic diversity of NLV strains we identified in this cohort during a short time period suggests multiple foci of infection within the community. Furthermore, sequence analysis of strains from two children with multiple symptomatic NLV infections indicates that infection with one strain was not protective against subsequent infection with a different strain in the same genogroup. These findings have implications for vaccine development and the prevention of pediatric gastroenteritis in developing countries. ***J. Med. Virol. 58:426–434, 1999.***

KEY WORDS: human calicivirus; small round structured virus; gastroenteritis; diarrhea

INTRODUCTION

Pediatric gastroenteritis is a major cause of childhood morbidity and mortality worldwide. Repeated episodes of acute diarrhea and vomiting or persistent symptoms can not only decrease quality of life, but also contribute to dehydration and malnutrition, and have adverse effects on development and survival. Children under 5 years of age in developing countries experience the highest rates of illness and death due to diarrhea, with the majority of disease occurring in infants under 1 year of age [Lima and Guerrant, 1992]. Despite the importance of gastrointestinal disease as a cause of poor health in young children, many cases are of unknown etiology [Guerrant, 1994].

Previously referred to as "small round structured viruses" or "Norwalk-related viruses," "Norwalk-like viruses" (NLVs) are small (27–32 nm), single-stranded positive sense RNA viruses recently designated as a genus in the *Caliciviridae* family based on phylogenetic analyses and genomic organization [Green et al., 1999]. NLVs have been divided into two genetically and antigenically distinct genogroups [Ando et al., 1994; Wang et al., 1994; Pringle, 1998]. Enteric infections with NLVs are associated with acute, self-limited symptoms of nausea, vomiting, and diarrhea, which may be accompanied by fever, headache, or myalgia. These viruses are highly infectious and are transmitted by environmental exposures such as fecally contaminated food, water, and shellfish, as well as directly from person-to-person [Kapikian et al., 1996].

Knowledge about the role of NLVs in pediatric gas-

Work on this project was performed at the Department of Epidemiology, School of Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC.

Grant sponsor: National Institute for Environmental Health Sciences.

*Correspondence to: Dr. Christine L. Moe, Department of Epidemiology, School of Public Health, CB# 7400, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7400.

Accepted 4 January 1999

troenteritis in developing countries has been limited by the lack of sensitive and specific diagnostic techniques. Infection rates in young children and infants living in developing countries may have been underestimated in previous studies that sought to identify viral particles in stool specimens by electron microscopy [Singh et al., 1989; Timenetsky et al., 1993; Paje-Villar et al., 1994]. The development of a recombinant antigen enzyme-linked immunosorbent assay (ELISA) has enabled studies of the sero-epidemiology of pediatric NLV infection in developing countries, and has led to an accumulation of evidence that these viruses commonly infect young children and infants. Several recent studies document a high, age-dependent seroprevalence in young children, suggesting early and repeated exposure [Gray et al., 1993; Jiang et al., 1995; Parker et al., 1995; Dimitrov et al., 1997; Smit et al., 1997]. NLVs have been detected by reverse transcription-polymerase chain reaction (RT-PCR) in children worldwide, including Mexico and South Africa [Jiang et al., 1995; Wolfaardt et al., 1995, 1997; Smit et al., 1997]. Although the sequences of several of these strains have been described, the extent of genetic diversity and epidemiology of NLVs in these populations remains to be understood.

Recent work in our laboratory indicated a high rate of seroconversion to recombinant Norwalk virus antigen in a cohort study of young children living in an urban shantytown in Brazil [unpublished]. To further investigate the epidemiology of NLV infection in this cohort, we screened stool specimens by RT-PCR. The goal of this study was to sequence the NLV-positive PCR products and to examine the genomic diversity of NLV strains detected in the study children over a 16-month period of follow-up. In addition, we sought to identify whether repeat or chronic NLV infections occurred in children who had multiple PCR-positive stool specimens several months apart.

METHODS

Stool Specimens

The sequencing templates used in this study were RT-PCR products derived from stool specimens collected in two studies of pediatric diarrhea in northeastern Brazil. Stool specimens were collected from a family-based cohort (GDI) of 186 children living in a four-block region of an urban shantytown in Forteleza (population 2 million), Ceará, Brazil. Living conditions and health outcomes in the shantytown cohort have been described previously [Schorling et al., 1990]. Children were enrolled at birth and followed until 5 years of age or the end of the study. During this time, they were visited three times per week by health workers who interviewed the caregivers regarding whether the children had experienced diarrhea or vomiting since the previous visit. An episode of acute diarrhea was defined as at least 1 day of increased stool frequency separated from other episodes by at least 3 days of nondiarrheal stools. Case specimens were collected during the diarrheal phase of every episode if possible, and control specimens were collected every 3–6 months. Stool specimens available for this study were a sample of all specimens collected from children with and without diarrhea between May 1990 and September 1991. Specimens were selected if they were positive for few or no other pathogens and had sufficient quantity for RNA extraction and testing. For two children with PCR-positive specimens, additional stool specimens preceding or following the date of the original positive specimen were obtained for testing.

Additional stool specimens were obtained from children hospitalized for persistent diarrhea (PDAS) at the Hospital Albert Sabin, the major pediatric hospital in Forteleza [Fang et al., 1995]. These specimens were collected between August 1988 and March 1991 from children under 3 years of age who were experiencing symptoms of diarrhea lasting more than 2 weeks. Specimens were collected during or within 3 days of the symptoms of persistent diarrhea.

Stool specimens were tested elsewhere as described for the presence of other viral pathogens (rotavirus, adenovirus, and torovirus), pathogenic bacteria (enterotoxigenic, enteropathogenic, enteroinvasive, and enteroadherent coliforms, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Aeromonas hydrophila*), and parasites (*Giardia lamblia, Entamoeba histolytica, Strongyloides stercoralis,* and *Cryptosporidium* spp.) [Schorling et al., 1990; Fang et al., 1995; Koopmans et al., 1997].

Origins of Sequencing Templates

Sequencing templates were RT-PCR products generated by either of two previously described primer systems that target a portion of the viral RNA polymerase gene for Norwalk virus and other Genogroup I and II NLVs (NV3 and NV51; G1 and G2) [Moe et al., 1994; Ando et al., 1995]. Initially, 159 stool specimens (123 GDI and 36 PDAS) were screened by RT-PCR utilizing the NV3 and NV51 primers. Nine strong positives from this screening were chosen for sequencing. Due to the limited availability of RNA, only a subset of GDI specimens ($n = 53$) were retested with the G1 and G2 primers. These specimens included some which yielded strong positive PCR products using the NV 3 and NV51 primers, as well as specimens that had weak or equivocal amplification products in the initial screening. All moderate to strong positive PCR products generated by the G1 and G2 primers were chosen as sequencing templates.

RT-PCR

Viral RNA was extracted from 300 μl of 20% stool suspensions utilizing a polyethylene glycol-cetyltrimethylammonium bromide precipitation protocol, and suspended in a final volume of 20 μl water [Jiang et al., 1992]. Purified RNA was used immediately for NV3 and NV51 RT-PCR reactions, and was stored at –80°C prior to amplification by the G1 and G2 primers. Reaction conditions for RT-PCR using the NV3 and NV51 primers and the G1 and G2 primers have been de-

scribed previously [Moe et al., 1994; Ando et al., 1995]. Positive and negative controls were run with every RNA extraction and RT-PCR assay. The Genogroup I-positive control was a stool specimen from a volunteer infected with Norwalk virus, and the Genogroup II-positive control was a stool specimen from an outbreak of a Genogroup II NLV. Negative controls contained equal volumes of water in place of RNA. Ten microliters of each PCR product was run on a 3% agarose gel with a size marker and visualized by staining with ethidium bromide (EtBr) and exposure to ultraviolet (UV) light. An NLV-positive stool sample was defined as one with a visible PCR product of the appropriate size (206 nt for primers NV3 and NV51, 123 nt for primers G1 and G2).

Sequencing Reactions

Double-stranded DNA templates were purified for sequencing using the QIAquick™ column purification kit (QIAGEN, Chatsworth, CA). If samples had a strong band of the appropriate size, but also extraneous bands due to nonspecific amplification, the template was isolated from a gel fragment as follows. Between 40 and 80 μl of PCR product was run on a 3% agarose gel, visualized by EtBr staining and UV exposure, and the band of the appropriate size isolated by excision from the gel prior to passage through the column. Samples with a strong, single band of the appropriate size were removed from under the mineral oil of the PCR reaction for direct passage through the column. Sequences were generated using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase FS using the manufacturer's protocol (Perkin-Elmer, Applied Biosystems Division, Foster City, CA). Sequencing primers were either the sense or antisense primers used for PCR. Sequences were generated in both directions to derive a consensus sequence for each strain. Sequencing reactions were run on an ABI Automated Sequencer using a 6% denaturing acrylamide gel (10:1 acrylamide: bis-acrylamide) and electrophoresed at 2,500 V. Data were collected and analyzed using ABI 373 software.

Sequence Analysis

The sequences of all the PCR products from NLV-positive samples were compared across an 81-nucleotide region internal to the G1 and G2 primers. Consensus sequences were derived for each PCR product. A strain was defined as a sequence that differed from any other sequence in the study at one or more nucleotide positions. Percentages of sequence identity were determined by computing the exact matches between the nucleotide or amino acid sequences for each pairwise comparison of strains. The GCG suite of programs [Wisconsin Package Version 9.1, Genetics Computer Group, Madison, WI] and PAUP [Swofford, 1991] were used to align and compare the strains identified in this study population with each other and with prototype strains. Nucleotide sequences for strains identified in this study (BraV1–8) were compared with prototype strains representing the two NLV genogroups: Norwalk virus (M87661), Mexico virus (U22498), Lordsdale virus (X86557), Desert Shield virus (U04469), Southampton virus (L07418), Snow Mountain virus (L23831), and Hawaii viruses (U07611) [Jiang et al., 1990, 1995; Lambden et al., 1993; Lew et al., 1994a; Wang et al., 1994; Dingle et al., 1995]. A basic local alignment search tool (BLAST) [Altschul et al., 1990] was used to identify sequences in GenBank with the greatest homology to each of the strains identified in this study.

Serologic Characterization

Serum immunoglobulin G (IgG) responses to Norwalk virus in cohort samples were measured using an enzyme immunoassay with recombinant Norwalk virus antigen (Monroe et al., 1993).

RESULTS

PCR and Sequencing

Fifteen NLV consensus sequences were identified in the stool specimens of 12 children (single specimens from 10 children and multiple specimens from two children) (Table I). Analysis of these sequences described eight unique strains that we subsequently designated as Brazil virus one through eight (BraV1–BraV8) (Tables I and II). Nucleotide sequence identity ranged from 63% to 88% between the eight strains (Table III). None of the strains identified in either the shantytown cohort or the hospital study were exactly homologous to any sequences previously described in GenBank, although nucleotide identity with strains in the database ranged from 85% to 98% (Table II). Peptide identity was slightly higher due to the presence of synonymous mutations. Peptide identity between the eight strains ranged from 67% to 100% (Table III) and was between 96% and 100% when compared with sequences in GenBank (data not shown).

Multiple sequence alignment and phylogenetic analyses indicated that the strains identified in this study could be classified within the NLV genogroups I and II described previously (Figs. 1 and 2; Table II) [Ando et al., 1994; Wang et al., 1994; Green et al., 1998]. Strains BraV1–3, BraV5, and BraV7 were related to the Genogroup I viruses, characterized by the prototype strains Norwalk virus, Desert Shield virus, and Southampton virus, whereas BraV4, BraV6, and BraV8 were more closely related to the Genogroup II strains, represented by Lordsdale virus, Mexico virus, Snow Mountain virus and Hawaii virus (Figs. 1 and 2). The five Genogroup I strains spanned the range of genetic diversity represented by the prototype strains, whereas the three Genogroup II strains were most closely related to Lordsdale virus (Fig. 2). The relation-

TABLE I. Description of the Stool Specimens, Clinical Features, and the NLV Strains Detected

Child[a]	Age	Date[b]	Diarrhea[c]	Vomiting (days)	PCR primers[d]	Strain	Other pathogens[e]
A	9 months	06/12/90	Acute (day 4)	4	G1, NV3/51	BraV2	EAggEC
A	13 months	11/20/90	Acute (day 0)	None	NV3/51	BraV3	None
B	5 weeks	05/31/90	Acute (day 1)	2	NV3/51	BraV2	EAggEC
B	4 months	08/28/90	Acute (day 3)	3	NV3/51	BraV3	None
B	7 months	12/03/90	Acute (day 5)	5	NV3/51	BraV5	LTEC STEC
C	6 months	07/22/91	Acute (day 3)	9	G2	BraV4	None
D	11 months	09/02/91	Acute (day 4)	11	G2	BraV4	EAggEC
E	13 months	06/26/91	Acute (day 1)	4	NV3/51	BraV2	None
F	3 months	06/18/90	None	None	G1	BraV2	EAggEC
G	11 months	12/05/90	Acute (day 4)	5	G2	BraV6	EAggEC
H	5 months	08/21/90	Acute (day 3)	4	NV3/51	BraV7	EPEC
I	7 months	06/18/90	None	None	G1, NV3/51	BraV2	None
J	5 months	07/03/91	None	None	G1	BraV1	None
PDAS1	11 months	02/06/91	Persistent	Unknown	NV3/51	BraV1	FAEC EPEC
PDAS2	5 months	07/04/89	Persistent	Unknown	G2	BraV8	EAggEC torovirus

NLV, Norwalk-like virus; PCR, polymerase chain reaction.
[a]From the GDI cohort unless PDAS.
[b]Date of specimen collection.
[c]Day of specimen collection relative to onset of acute diarrhea in parentheses.
[d]Primers used to generate sequencing template(s).
[e]EAggEC, Enteroaggregative *E. coli*; LTEC, heat labile enterotoxigenic *E. coli*; STEC, heat stabile enterotoxigenic *E. coli;* EPEC, attaching and effacing *E. coli*; FAEC, focal adherent *E. coli*.

ships between the eight Brazil strains were slightly altered when the amino acid sequences were compared, but they retained their genogroup assignments (results not shown).

Molecular Epidemiology

Thirteen stool samples from 10 children in the shantytown cohort were sequenced, and yielded seven unique strains (Table I). The age of the infected children ranged from 5 weeks to 13 months, and male and female children were equally represented. Ten of the 13 GDI specimens were obtained from children with acute episodes of diarrhea, and 9 of these 10 samples were also associated with symptoms of vomiting lasting between 2 and 11 days in duration. Three specimens were collected from children with no reported symptoms of either diarrhea or vomiting. Pathogenic *Escherichia coli* were detected in 7 of the 13 acute diarrhea stool specimens and both persistent diarrhea stool specimens.

The isolation period of the NLV strains detected in the shantytown cohort was over 16 months. One strain, BraV2, was detected in a cluster of four specimens collected in May and June of 1990, and was identified again 1 year later in a single case. The remaining strains were detected sporadically during the study period. Two PCR products from the hospitalized children with persistent diarrhea were sequenced. One yielded a strain (BraV1) that was identical to that detected in one of the cohort specimens, and the other strain (BraV8) was different from all other strains identified in the study. The hospital isolate BraV1 was collected early in 1991, a time that overlapped with the collection of cohort specimens, whereas the specimen that yielded the unique BraV8 strain was collected 6 months before the first cohort sample we tested.

Two children in the shantytown cohort experienced multiple episodes of acute diarrhea associated with different strains (Table I). Child A, at age 9 months, had an acute episode of diarrhea and vomiting associated with BraV2. Three months later, he had another episode of diarrhea associated with BraV3. Child B also was infected with these same two strains in succession. At age 5 weeks, Child B experienced an episode of acute diarrhea and vomiting associated with BraV2, and 11 weeks later, at age 4 months, he had another acute episode of diarrhea and vomiting associated with BraV3. This second episode was followed by 4 weeks of persistent diarrhea (additional stool specimens tested), and 3 months later, he experienced another acute epi-

TABLE II. Characteristics of the Brazil NLV Strains

Strain[a]	Primers[b]	Genogroup[c]	Closest match[d] (% identity)	Origin of closest match[e]
BraV1	G1, NV3/51	I	SRSVRNAP9 (96%) Z29479	UK—unspecified Norcott, 1994
BraV2	G1, NV3/51	I	UK2/12121/89/UK (84%) S71765	UK—adult, sporadic Ando et al., 1994
BraV3	NV3/51	I	Saratoga virus 7 (98%) U07614	Military ship Lew et al., 1994a
BraV4	G2	II	Camberwell virus (95%) U46500	Australia-outbreak Cauchi et al., 1996
BraV5	NV3/51	I	UK2/12121/89 (94%) S71765	UK—adult, sporadic Ando et al., 1994
BraV6	G2	II	Bristol virus (91%) X76716	UK—outbreak Green et al., 1994
BraV7	NV3/51	I	Sa-1283/84/J (95%) L23832	Japan—infant, sporadic Wang et al., 1994
BraV8	G2	II	NCRNAPBS1 (93%) Y13706	Germany—unspecified Direct submission, 1997

NLV, Norwalk-like virus.
[a]Unique strain identified in the study.
[b]Polymerase chain reaction product used as sequencing template.
[c]Genogroup classification based on dendrogram.
[d]Most closely related strain in GenBank (% nucleotide identity) as identified by the basic local alignment search tool (BLAST) and GenBank accession number.
[e]For each strain identified by BLAST, the country of origin, source of sample (pediatric or adult, outbreak or sporadic case), and author and year of publication or direct submission to GenBank.

TABLE III. Sequence Identity (%) in the RNA Polymerase Region Among Brazil NLVs and Reference Strains

	Genogroup I						Genogroup II			
	NV[a]	BraV1	BraV2	BraV3	BraV5	BraV7	SMA[a]	BraV4	BraV6	BraV8
NV		74	65	78	78	89	62	60	63	62
BraV1	93		77	75	77	76	72	76	64	65
BraV2	89	96		79	77	69	65	64	63	63
BraV3	93	100	96		77	74	69	65	64	68
BraV5	93	100	100	100		74	64	65	68	68
BraV7	100	93	89	93	93		67	67	68	67
SMA	78	81	85	81	81	78		82	83	81
BraV4	81	85	81	85	85	81	96		88	86
BraV6	81	85	81	85	85	81	96	100		88
BraV8	78	81	78	81	81	78	93	93	96	

Numbers on top-right indicate results of pairwise comparison of 81 nucleotide region of RNA polymerase gene. Numbers on bottom-left indicate results of pairwise comparison of 27 peptide translation of the same region. Sequence identity is the percentage of exact matches of nucleotides or amino acids.
[a]NV, Norwalk virus, reference strain for Genogroup I; SMA, Snow Mountain virus, reference strain for Genogroup II.

sode of diarrhea and vomiting associated with a third strain, BraV5.

Serology

Results of serologic testing for antibodies to Norwalk virus (NV-IgG) were evaluated for the two children who experienced multiple episodes of NLV-associated gastroenteritis. Levels of NV-IgG were elevated (50,200 units) in Child A 2 weeks after an acute episode of gastroenteritis associated with BraV2. They remained high (59,000 units) when measured 1 month before a second episode associated with BraV3. In Child B, no NV-IgG antibodies (<200 units) were detected at age 5 months after two NLV-associated diarrhea episodes, but NV-IgG was slightly elevated (3,800 units) 1 year later.

DISCUSSION

NLVs are a common cause of epidemic gastroenteritis in adults and older children in developed countries. However, little is known about the role of NLVs in endemic pediatric gastroenteritis in developing countries. We observed a high degree of genomic diversity in the NLVs circulating among a cohort of infants and young children in an urban Brazilian shantytown. We identified seven unique strains in 13 PCR-positive stool specimens collected from a cohort of 186 children over a 16-month period. The extent of NLV genomic diversity identified in this cohort was unexpected, but is consistent with a growing body of evidence of genetic and antigenic diversity in this group of viruses. [Kapikian et al., 1996; Noel et al., 1997]. Based on recent data from the US and UK, there appears to be a

```
        1                                                  50                                  81
    DSV ATTCTCACCC TGTGTGCATT GTCAGAAGTC ACCGGGCTCT CCCCAGATGT GTTGCAGTCA CAGTCGTATT TTTCCTTCTA T
  BraV3 atcctaactc tatgtgcatt gtcagaagtc actggcttgt cccctgatgt gatacaatca caatcttatt tctcatttta t
  BraV5 ctaattaccc tttgtgccct ctctgaggtc actgggctct cccctgatgt aatacagtca cagtcatatt tttcatttta t
  BraV2 atcttaacct tgtgcgccct gtcagaagtt actggtctgg ctcctgatgt aatacagtca caatcttact tttcattcta t
Norwalk ATAATTACTC TCTGTGCACT GTCTGAGGCC ACTGGTTTAT CACCTGATGT GGTGCAATCC ATGTCATATT TCTCATTTTA T
  BraV7 ataatcaccc tttgtgcact gtctgaggct actggcttgt cacctgacgt ggtgcaatcc atgtcatact tctcattcta t
    SOH TTAATAACTC TGTGCGCCCT TTCTGAAGTG ACTGGCCTGT CGCCAGACGT TATCCAATCC ATGTCATATT TCTCTTTCTA T
  BraV1 ttaatcacct tgtgtgccat gtctgaagtc acaggcctat cacctgatgt catacaatcc cagtcttact tctctttcta t
Cons-G1 -T--T-AC-- T-TGTGC--T -TC-GA-G-- AC-GG--T-- C-CC-GA-GT --T-CA-TC- ---TC-TA-T T-TC-TT-TA T

Cons-G2 -T-CT-AC-C T-TGTGC-CT -T-TGA-GT- AC----CT-- --CC-GACAT -AT-CA-GC- AA-TC--T-T --TC-TT-TA -
     LV CTCCTCACTC TCTGTGCACT CTCTGAAGTT ACAAACCTGT CCCCTGACAT CATTCAGGCT AATTCCCTCT TTTCCTTCTA T
  BraV4 ctcctcactc tttgtgcact ctctgaagtt acaaatctgt cccctgacat tattcaggct aattccctct tctccttcta c
  BraV6 ctcctcactc tctgtgcact atctgaggtt acaaacctct cccctgacat tatccaggcc aactccctct tttccttcta t
  BraV8 ctcctcacac tctgtgcact ctttgaggtt acaaacctgt cccctgacat catccaggca aattctctct tctcctttta t
    SMA CTCCTCACAC TCTGTGCACT ATCTGAAGTC ACAAACCTGG CTCCTGACAT CATACAAGCT AACTCCTTGT TCTCTTTCTA T
 Hawaii CTCCTCACTC TTTGTGCACT CTCTGAAGTC ACGGACCTGT CCCCTGACAT CATTCAAGCC AATTCCTTAT TCTCTTTCTA T
 Mexico TTGCTTACTC TGTGTGCCCT TTCTGAAGTG ACAGGACTAG GCCCCGACAT CATACAAGCT AATTCTATGT ACTCTTTCTA T
```

Fig. 1. Nucleotide alignment of Brazil Strains 1–8 compared with seven reference strains. Multiple sequence alignment of an 81 nucleotide region of the viral RNA polymerase gene from Brazil strains 1–8, compared with known reference strains (Norwalk; DSV, Desert Shield; SOH, Southampton; LV, Lordsdale; SMA, Snow Mountain, Hawaii, Mexico). Position one corresponds to nucleotide 4774 of the Norwalk virus genome. Sequences are grouped into two clusters by the GCG Pileup program, the upper eight in Genogroup I, and the lower seven in Genogroup II. Cons-G1 and G2 indicate consensus sequences for Genogroups I and II, respectively. Lower case letters indicate the sequences of Brazil strains 1–8, and upper case letters indicate the reference strain and consensus sequences.

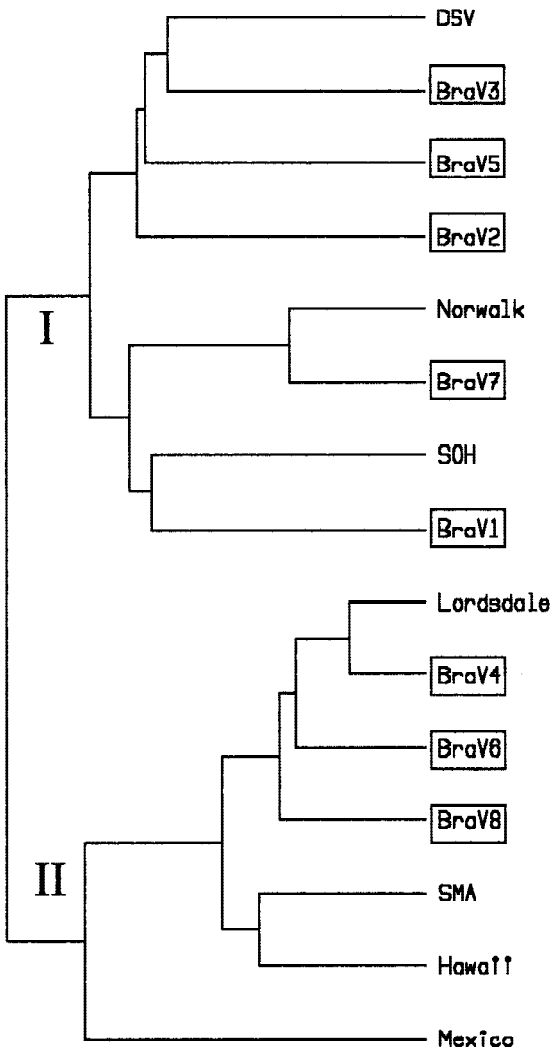

Fig. 2. Dendrogram showing the clustering relationships used to generate multiple sequence alignment of the 81 nucleotide portion of the viral RNA polymerase gene (corresponding to bases 4774–4865 of Norwalk virus) of Brazil strains 1–8 with other known Genogroup I and II strains (Norwalk; DSV, Desert Shield; SOH, Southampton; Lordsdale; SMA, Snow Mountain, Hawaii, Mexico). Generated using GCG Pileup program.

wider diversity of strains circulating among children in this cohort than that observed in developed countries (Maguire et al., 1998; Noel et al., 1998).

Our study builds on previous research on pediatric NLV infection by examining strains detected in a small cohort of children over a short duration. Because our samples came from a relatively small cohort defined by geographical and socioeconomic characteristics, our results provide a foundation for understanding the molecular epidemiology of pediatric NLV infection in developing countries. A similar study conducted in a "peri-urban" population in Mexico reported a high seroprevalence in young children, and described the sequence of one strain collected from a sick child [Jiang et al., 1995]. Two recent studies have described several genetic sequences of NLVs from pediatric cases in South Africa [Smit et al., 1997; Wolfaart et al., 1997], however most were sporadic cases seen at hospitals serving large geographic areas.

The high number of unique strains we detected, relative to the number of specimens we examined and the short time period of specimen collection, suggests that there were multiple foci of infection in this community. This phenomenon has been observed previously with polioviruses, in which genomic heterogeneity of strains identified in a community reflects the presence of many independent reservoirs that maintain endemicity [Kew et al., 1990]. Our findings reported here support serological data indicating early and multiple NLV infections in the children of this cohort [data not shown], and suggest that NLVs may follow endemic patterns of transmission in this population. This pattern may be attributed to the poor sanitation and hygiene observed in this community and early introduction of supplementary foods to the diets of infants. Previous studies have documented high rates of diarrheal disease and infection with many other enteric pathogens in this population (Schorling et al., 1990; Fang et al., 1995).

Our findings contrast with several recent studies that identified a preponderance of Genogroup II NLV infections in pediatric cases from both developed and developing countries [Lew et al., 1994b; Jiang et al., 1995; Parker et al., 1995; Wolfaardt et al., 1997; Wright et al., 1998]. We identified twice as many Genogroup I strains ($n = 6$) than Genogroup II ($n = 3$) in our cohort and hospital specimens. However, our initial RT-PCR screening using the NV3 and NV51 primer pair (based on the Norwalk virus genome) may have preferentially detected Genogroup I strains. Genogroup I and II strains were detected with equal frequency using the G1/G2 primers. None of the strains detected in this study shared high homology to Toronto virus or Mexico virus, two very similar Genogroup II strains identified in cases of pediatric gastroenteritis in Canada and Mexico [Lew et al., 1994b; Jiang et al., 1995]. The potential for drawing inferences about the relative prevalence of Genogroup I and Genogroup II infections from these data and other studies that sequenced only a few strains is limited due to the small number of samples.

In the two children (Child A and B) with multiple NLV PCR-positive specimens, infection with one strain was not protective against subsequent infection with another strain within the same genogroup. Both of these children were first infected with BraV2 followed by BraV3, two similar Genogroup I strains with a high level of peptide identity in the region of the polymerase gene we examined. Child B was infected 3 months later with a third Genogroup I strain (BraV7). In Child A, IgG antibodies to Norwalk virus were elevated on two separate occasions after infection with BraV2, which suggests that a strong NV-IgG response to one Genogroup I strain did not protect this child against infection from a second Genogroup I strain. The lack of an apparent immune response in Child B may be explained by an immature immune system. Immunity to NLV infection in adults is poorly understood (Kapikian et al., 1996), and experimental studies have not been

conducted in children. Human challenge studies with Norwalk and Hawaii virus have demonstrated that short-term immunity is serotype specific, but that IgG response does not confer long-term immunity against a repeat viral challenge with the same strain (Wyatt et al., 1974; Parrino et al., 1977). Other studies with adult volunteers indicate that the presence of anti-NV IgG may predispose an individual to reinfection or be a marker of susceptibility to infection (Johnson et al., 1990; Gray et al., 1993; Moe et al., 1998). It is not known whether these observations extend to other NLV strains or to immunity in children. Our results suggest that young children either with or without anti-NV IgG may be susceptible to repeated symptomatic NLV infection. Vaccine strategies to prevent pediatric NLV infections in developing countries will need to consider the problem of frequent exposures to different NLV strains.

Frequent symptomatic NLV infections in infants and young children living in developing countries may impact quality of life substantially and contribute to dehydration and malnutrition. Although based on a limited number of specimens, the genomic diversity of NLVs we observed suggests an endemic pattern of NLV transmission within this population. Further information on the molecular epidemiology of NLV infections, transmission patterns, and immunity is needed to support the development of effective public health interventions to reduce pediatric NLV disease in developing countries.

ACKNOWLEDGMENTS

The authors are grateful to David Fenstermacher and Helen Sun for their technical assistance. This work was supported in part by the National Institute for Environmental Health Sciences Predoctoral Training Grant in Environmental Epidemiology.

REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403–410.

Ando T, Monroe SS, Gentsch JR, Jin Q, Lewis DC, Glass RI. 1995. Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and Southern hybridization. J Clin Microbiol 33:64–71.

Ando T, Mulders MN, Lewis DC, Estes MK, Monroe SS, Glass RI. 1994. Comparison of the polymerase region of small round structured virus strains previously classified in three antigenic types by solid-phase immune electron microscopy. Arch Virol 135:217–226.

Cauchi MR, Doultree JC, Marshall JA, Wright PJ. 1996. Molecular characterization of Camberwell virus and sequence variation in ORF3 of small round-structured (Norwalk-like) viruses. J Med Virol 49:70–76.

Dimitrov DH, Dashti SA, Ball JM, Bishbishi E, Alsaeid K, Jiang X, Estes MK. 1997. Prevalence of antibodies to human caliciviruses (HuCVs) in Kuwait established by ELISA using baculovirus-expressed capsid antigens representing two genogroups of HuCVs. J Med Virol 51:115–118.

Dingle KE, Lambden PR, Caul EO, Clarke IN. 1995. Human enteric *Caliciviridae:* the complete genome sequence and expression of virus-like particles from a genetic group II small round structured virus. J Gen Virol 76:2349–2355.

Fang GD, Lima AA, Martins CV, Nataro JP, Guerrant RL. 1995. Etiology and epidemiology of persistent diarrhea in northeastern Brazil: a hospital-based, prospective, case-control study. J Pediatr Gastroenterol Nutr 21:137–144.

Gray JJ, Jiang X, Morgan-Capner P, Desselberger U, Estes MK. 1993. Prevalence of antibodies to Norwalk virus in England: detection by enzyme-linked immunosorbent assay using baculovirus-expressed Norwalk virus capsid antigen. J Clin Microbiol 31:1022–1025.

Green KY, Ando T, Balayan MS, Estes MK, Maston DO, Nakata S, Neil JD, Studdert MJ, Thiel HJ. 1999. Taxonomy of the caliciviruses. International Workshop on Human Caliciviruses. Centers For Disease Control, Atlanta, GA, USA. March 29–31.

Green SM, Dingle KE, Lambden PR, Caul EO, Ashley CR, Clarke IN. 1994. Human enteric *Caliciviridae:* a new prevalent small round-structured virus group defined by RNA-dependent RNA polymerase and capsid diversity. J Gen Virol 75:1883–1888.

Guerrant RL. 1994. Twelve messages from enteric infections for science and society. Am J Trop Med Hyg 51:26–35.

Jiang X, Matson DO, Velazquez FR, Calva JJ, Zhong WM, Hu J, Ruiz-Palacios GM, Pickering LK. 1995. Study of Norwalk-related viruses in Mexican children. J Med Virol 47:309–316.

Jiang X, Wang J, Grahm DY, Estes MK. 1992. Detection of Norwalk virus in stool by polymerase chain reaction. J Clin Microbiol 30: 2529–2534.

Johnson PC, Mathewson JJ, DuPont HL, Greenberg HB. 1990. Multiple-challenge study of host susceptibility to Norwalk gastroenteritis in US adults. J Infect Dis 161:18–21.

Kapikian AZ, Estes MK, Chanock RM. 1996. Norwalk group of viruses. In: Fields BN, Knipe DM, Howley PM, editors. Fields virology, 3rd ed. Philadelphia: Lippincott–Raven Publishers. p 783–810.

Kew OM, Nottay BK, Rico-Hesse R, Pallansch MA. 1990. Molecular epidemiology of wild poliovirus transmission. In: Kurstak E, Marusyk RG, Murphy FA, Van Regenmortel MHV, editors. Applied virology research, vol. 2, virus variability, Epidemiology and control. New York: Plenum Medical Book Co., p 199–221.

Koopmans MP, Goosen ES, Lima AA, McAuliffe IT, Nataro JP, Barrett LJ, Glass RI, Guerrant RL. 1997. Association of torovirus with acute and persistent diarrhea in children. Pediatr Infect Dis J 16:504–507.

Lambden PR, Caul EO, Ashley CR, Clarke IN. 1993. Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science 259:516–519.

Lew JF, Kapikian AZ, Valdesuso J, Green KY. 1994a. Molecular characterization of Hawaii virus and other Norwalk-like viruses: evidence for genetic polymorphism among human caliciviruses. J Infect Dis 170:535–542.

Lew JF, Petric M, Kapikian AZ, Jiang X, Estes MK, Green KY. 1994b. Identification of Minireovirus as a Norwalk-like virus in pediatric patients with gastroenteritis. J Virol 68:3391–3396.

Lima AAM, Guerrant RL. 1992. Persistent diarrhea in children: epidemiology, risk factors, pathophysiology, nutritional impact, and management. Epidemiol Rev 14:222–241.

Maguire AJ, Green J, Brown DWG, Desselberger U, Gray JJ. 1998. Molecular epidemiology of gastroenteritis caused by small round structured viruses (SRSV) in East Anglia, UK. Proceedings of the 17th Annual Meeting of the American Society for Virology, Vancouver, BC. Abstract 15-10, p. 182.

Moe CL, Gentsch J, Ando T, Grohmann G, Monroe SS, Jiang X, Wang J, Estes MK, Seto Y, Humphrey C, Stine S, Glass RI. 1994. Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis. J Clin Microbiol 32:642–648.

Moe C, Rhodes D, Pusek S, Tseng F, Heizer W, Kapoor C, Gilliam B, Harb M, Stewart P, Miller S, Sobsey M, Hermann J, Blacklow N, Calderon R. 1998. Determination of Norwalk virus dose–response in human volunteers. Proceedings of the 98th General Meeting of the American Society for Microbiology, Atlanta, GA. Abstract C-384.

Monroe SS, Stine SE, Jiang X, Estes MK, Glass RI. 1993. Detection of antibody to recombinant Norwalk virus antigen in specimens from outbreaks of gastroenteritis. J Clin Microbiol 31:2866–2872.

Noel JS, Ando T, Leite JP, Green KY, Dingle KE, Estes MK, Seto Y, Monroe SS, Glass RI. 1997. Correlation of patient immune response with genetically characterize small round-structured viruses involved in outbreaks of nonbacterial acute gastroenteritis in the United States, 1990 to 1995. J Med Virol 53:372–383.

Noel JS, Frankhauser RL, Ando T, Monroe SS, Glass RI. 1998. The dynamics of small round structured virus infections: probably emergence of a global strain. Proceedings of the 17th Annual

Meeting of the American Society for Virology, Vancouver, BC. Abstract P15-9, p. 182.

Norcott JP, Green J, Lewis D, Estes MK, Barlow KL, Brown DWG. 1994. Genomic diversity of small round structural viruses in the United Kingdom. J Med Virol 44:280–286.

Paje-Villar E, Co BG, Caradang EH, Raymundo A, Lagamayo E, Lavadia E, Rigor E, Hart CA. 1994. Non-bacterial diarrhea in children in the Philippines. Ann Trop Med Parasitol 88:53–58.

Parker SP, Cubitt WD, Jiang X. 1995. Enzyme immunoassay using baculovirus-expressed human calicivirus (Mexico) for the measurement of IgG responses and determining its seroprevalence in London, UK. J Med Virol 46:194–200.

Parrino TA, Schreiber DS, Trief JS, Kapikian AZ, Blacklow NR. 1977. Clinical immunity in acute gastroenteritis caused by Norwalk agent. N Engl J Med 297:86–89.

Pringle CR. 1998. Virus taxonomy—San Diego 1998. Arch Virol 143: 1449–1459.

Schorling JB, Wanke CA, Schorling SK, McAuliffe JF, deSouza MA, Guerrant RL. 1990. A prospective study of persistent diarrhea among children in an urban Brazilian slum. Am J Epidemiol 132: 144–156.

Singh BP, Sreenivasan MA, Pavri KM. 1989. Viruses in acute gastroenteritis in children in Pune, India. Epidemiol Infect 102:345–353.

Smit TK, Steele AD, Peenze I, Jiang XI, Estes MK. 1997. Study of Norwalk virus and Mexico virus infections at Ga-Rankuwa, South Africa. J Clin Microbiol 35:2381–2385.

Swofford DL. 1991. PAUP: Phylogenetic analysis using parsimony, version 3.s. Champaign, IL: Illinois Natural History Survey.

Timenetsky M, Kisielius JJ, Grisi SJ, Escobar AM, Ueda M, Tanaka H. 1993. Rotavirus, adenovirus, astrovirus, calicivirus, and small round virus particles in feces of children with and without acute diarrhea, from 1987 to 1988, in the greater Sao Paulo. Revista do Instituto de Medicina Tropical de Sao Paulo 35:275–280.

Wang J, Jiang X, Madore HP, Gray J, Desselberger U, Ando T, Seto Y, Oishi I, Lew JF, Green KY, Estes MK. 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. J Virol 68:5982–5990.

Wolfaardt M, Taylor MB, Booysen HF, Engelbrecht L, Grabow WOK, Jiang X. 1997. Incidence of human calicivirus and rotavirus infection in patients with gastroenteritis in South Africa. J Med Virol 51:290–296.

Wolfaardt M, Taylor MB, Grabow WO, Cubitt WD, Jiang X. 1995. Molecular characterization of small round structured viruses associated with gastroenteritis in South Africa. J Med Virol 47:386–391.

Wright PJ, Gunesekere IC, Doultree JC, Marshall JA. 1998. Small round-structured (Norwalk-like) viruses and classical human caliciviruses in Southeastern Australia, 1980–1996. J Med Virol 55: 312–320.

Wyatt RG, Dolin R, Blacklow NR, DuPont HL, Buscho RF, Thornhill TS, Kapikan AZ, Channock RM. 1974. Comparison of three agents of acute infectious non-bacterial gastroenteritis by cross-challenge in volunteers. J Infect Dis 129:709–714.